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Does the Clearance of Inhaled ^{99m}Tc -Sestamibi Correlate with Multidrug Resistance Protein 1 Expression in the Human Lung?¹

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Purpose:

To examine the relation between the lung elimination rate of inhaled technetium ^{99m}Tc -sestamibi and immunohistochemical expression of bronchopulmonary multidrug resistance protein 1 (MRP1) and permeability glycoprotein (P-gp) and assess the repeatability of the inhaled ^{99m}Tc -sestamibi clearance technique.

Materials and Methods:

^{99m}Tc -sestamibi is a known substrate for P-gp and MRP1, which are established cellular drug efflux transporters. The elimination rate of ^{99m}Tc -sestamibi from the lungs after inhalation as an aerosol has been hypothesized to be regulated by expression of these transporters. Institutional ethics committee approval was received for this prospective study. Written informed consent was obtained from all participants. The clearance of inhaled ^{99m}Tc -sestamibi from the lungs of 13 patients due to undergo surgery for primary lung cancer (five of 13) or spontaneous pneumothorax (eight of 13) was estimated after dynamic imaging of the lungs during a period of 40 minutes. The time taken to clear 50% of inhaled sestamibi ($T_{1/2}$) was compared with a semiquantitative immunohistochemical assessment (grade 0–3) of MRP1 and P-gp expression in the lung by using parametric and nonparametric tests. The study was repeated in five participants to assess the repeatability of the technique by using a Bland Altman analysis method.

Results:

MRP1 expression was seen in 12 of 13 patients, while P-gp expression was seen in only two. The mean ^{99m}Tc -sestamibi elimination rate was faster in patients ($n = 6$) with low levels of MRP1 expression (grade 0–1) and mean $T_{1/2}$ of 105 minutes \pm 20 (standard deviation), compared with those with higher levels of MRP1 expression (grade 2–3, $n = 7$) and mean $T_{1/2}$ of 149 minutes \pm 28 ($P = .008$). Bland-Altman analysis revealed excellent agreement between test and retest values.

Conclusion:

Inhaled ^{99m}Tc -sestamibi clearance study is a repeatable technique demonstrating significant correlation with MRP1 expression in the lungs.

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Chemotherapy for lung cancer was introduced in the late 1960s and has resulted in an increase in median survival and long-term disease-free survival (1–3). Despite advances in cancer treatment, multidrug resistance to chemotherapy remains a major clinical problem resulting in poor patient outcome due to limited therapeutic options. The mechanisms of resistance development are often diverse and include insufficient uptake of water-soluble drugs by specific transporters, alterations in cellular pathways, which result in a reduced capacity of cytotoxic drugs to kill cells, or increased drug efflux from cancer cells mediated by cellular drug transporters.

Permeability glycoprotein (P-gp), a 170-kd ATP-dependent membrane transporter that acts as a drug efflux pump (4,5), was originally described by Juliano and Ling in 1976, and its role as a drug transporter in various clinical settings has been studied extensively (6–13).

Advances in Knowledge

- The results of the study indicate strong expression of multidrug resistance protein 1 (MRP1) (12 of 13) and weak (two of 13) or negative permeability glycoprotein expression in the human lungs.
- There was a significant correlation ($P = .0001$) between the clearance of inhaled technetium ^{99m}Tc -sestamibi and MRP1 expression in the lungs.
- Inhaled ^{99m}Tc -sestamibi clearance analysis is a safe technique, with radiation exposure of less than 0.5 mSv.
- Inhaled ^{99m}Tc -sestamibi clearance is a repeatable technique, with a bias of 3.23% (4.7 minutes) and a precision of 3.9% (4.7 minutes).
- Inhaled ^{99m}Tc -sestamibi clearance technique demonstrates significant interobserver correlation ($R = 0.97265$, two-tailed $P < .001$, and concordance correlation coefficient of 0.9865).

Multidrug resistance protein 1 (MRP1), identified by Cole and Deeley in 1992, has been the other most prominent protein that has been studied as a clinically relevant drug transporter (14–19) and has been closely linked to the development of clinical multidrug resistance in several types of cancer (16). MRP1 is widely expressed in many human tissues and cancers (15), and its role in the transport of an extraordinarily wide variety of substrates including a broad spectrum of anticancer drugs has been identified (14).

The radiopharmaceuticals technetium ^{99m}Tc -sestamibi and ^{99m}Tc -tetrofosmin, originally designed for myocardial perfusion studies, are well known substrates for both P-gp and MRP1 and have been described in the literature for the functional imaging of P-gp and MRP1 in humans (17–19). Many in vitro and in vivo studies have demonstrated correlation between the uptake and/or retention of these radiopharmaceuticals and the expression levels of P-gp and/or MRP1 in tumor cells (17,20–22). Therefore, ^{99m}Tc -sestamibi and ^{99m}Tc -tetrofosmin have been proposed as imaging probes for multidrug resistance assessment and to monitor the efficacy of multidrug resistance modulators (23,24). All these studies have studied the handling of the radiopharmaceutical after intravenous injection but none have specifically looked at the cellular handling of inhaled aerosols.

Recently, Ruparelia et al measured the clearance rate of ^{99m}Tc -sestamibi following inhalation as an aerosol from normal lungs in healthy nonsmoking humans (25). They observed that it was significantly slower than the clearance of the hydrophilic radioaerosol, ^{99m}Tc -pentetate (25), and hypothesized that the delayed clearance of ^{99m}Tc -sestamibi was due to expression of P-gp in the airway epithelium (26). The clearance rate of inhaled ^{99m}Tc -sestamibi from the lungs was further significantly delayed in healthy smokers versus nonsmokers, and it was hypothesized that this was the result of smoke-induced up-regulation of P-gp (25).

The aims of the study were to examine the relation between the lung elimination rate of inhaled ^{99m}Tc -sestamibi and immunohistochemical expression of bronchopulmonary MRP1 and P-gp and to assess the repeatability of the inhaled ^{99m}Tc -sestamibi clearance technique.

Materials and Methods

Institutional ethics committee approval was received for this prospective study. Written informed consent was obtained from all participants. The study was conducted in compliance with the principles of the Declaration of Helsinki (1996), the principles of Good Clinical Practice, and all of the applicable regulatory requirements.

Patients attending the specialist adult thoracic surgical clinic for spontaneous pneumothorax and lung cancer management were screened to identify eligible participants. The inclusion criteria included those aged 18 years or older and undergoing surgery for primary lung cancer or recurrent pneumothorax. They were all able to provide informed consent and able to lie supine for 40 minutes under the gamma camera. The exclusion criteria included patients who were younger than 18 years of age, pregnant, or if they were

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Abbreviations:

MRP1 = multidrug resistance protein 1

P-gp = permeability glycoprotein

Author contributions:

Guarantors of integrity of entire study, H.K.M., J.R.B., A.M.P.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; approval of final version of submitted manuscript, all authors; agrees to ensure any questions related to the work are appropriately resolved, all authors; literature research, H.K.M., J.R.B., A.M.P.; clinical studies, H.K.M., T.R., L.L., J.R.B., A.M.P.; experimental studies, H.K.M., P.C., L.L.; statistical analysis, H.K.M., L.L., A.M.P.; and manuscript editing, all authors

Conflicts of interest are listed at the end of this article.

receiving known P-gp/MRP1-modulating medication.

Nineteen potential patients were screened, of whom 13 (nine men) undergoing surgery for primary lung cancer (five of 13) or spontaneous pneumothorax (eight of 13) consented to the study. Of these, nine were cigarette smokers. Mean age (\pm standard deviation) for all the participants was 48 years \pm 24 (range, 19–78 years). Mean age of the male participants was 50 years \pm 25 (range, 19–78 years) and that for female patients was 45 years \pm 24 (range, 28–70 years). There was no statistically significant difference (*t* test, *P* = .36) in the mean ages between the male and female patients. All participants underwent inhaled ^{99m}Tc -sestamibi clearance study prior to the proposed surgery, and immunohistochemical examination of the resected lung tissue for MRP1 and P-gp expression was performed on the resected lung tissue. The inhaled ^{99m}Tc -sestamibi clearance study was repeated in five consecutive participants who consented to assess the repeatability of the technique. The repeat study was performed within 2–4 weeks of the initial study and prior to the planned surgery. The participants did not undergo any intervention between the two studies. There was also no alteration in the participant's physical condition between the two studies as assessed (H.K.M.) during interview prior to the imaging. The clearance rate of ^{99m}Tc -sestamibi from the lungs was correlated with a semiquantitative assessment (grade 0–3) of MRP1 and P-gp expression in lung tissue.

Inhaled ^{99m}Tc -Sestamibi Clearance Study Acquisition Parameters

A total of 400 MBq of ^{99m}Tc -sestamibi were placed in the SmartVent nebulizer (Diagnostic Imaging, Welford, Northants, United Kingdom). The nebulized radiopharmaceutical was inhaled for 1–5 minutes via a mouthpiece by the participant sitting upright until a minimum count rate was observed over the lungs, at least 0.3 counts per second. The varied inhalation time was to ensure that minimum count rates were achieved

by allowing for the differences in breathing abilities between participants.

Participants were positioned supine on a camera couch (Symbia; Siemens, Erlangen, Germany). Anterior and posterior images of the chest were acquired dynamically at a rate of 2 seconds per frame starting within a minute of completing the inhalation of the aerosol. Matrix size was 64×64 and no zoom was applied. The imaging continued up to 40 minutes. Images were transferred to Hermes (Hermes Medical, Stockholm, Sweden) workstation for analysis.

Analysis of Inhaled ^{99m}Tc -Sestamibi Clearance

Analysis of the dynamic images was performed by using Hermes workstation with the default dynamic study analysis software package. Regions of interest were drawn by an experienced (15 years) nuclear medicine physician (H.K.M.) around both lungs on the anterior and posterior images. Time-activity curves were generated for both lungs from the geometric mean of the data. While analysis of the clearance was performed only on the posterior views of the lungs in the previous study (25), we used a geometric mean from anterior and posterior view as this would provide a more accurate measure of the clearance, partly accounting for the depth-dependency of the activity distribution of the radiopharmaceutical and differential photon attenuation.

The effective half-time ($T_{1/2}$) of the elimination of activity from the lungs was calculated by using an exponential fit to the data. An exponential fit to the data was applied, while in the previous study by Ruparel et al (25) a linear fit was used. This was to avoid any assumptions, especially with the early part of the curve, which in the previous study (25) was not included. In this study, the imaging was commenced within a minute of completing inhalation, while in the previous study the imaging was commenced 6 minutes after completion of inhalation.

In five participants, the inhaled ^{99m}Tc -sestamibi clearance study was repeated within 2–4 weeks of the initial

study to assess the repeatability of the technique. A second experienced (6 years) nuclear medicine physician reanalyzed the inhaled ^{99m}Tc -sestamibi clearance in five patients to assess the correlation between the individual lung clearances and the mean clearance times.

Tissue Immunohistochemistry

Paraffin-embedded lung tissue obtained from the participants who underwent surgery for underlying disease was subjected to immunohistochemical examination for the presence of P-gp and MRP1. Immunohistochemistry was performed by using a staining machine (Ventana BenchMark ULTRA; Ventana Medical Systems, Tuscon, Ariz). Immunostaining procedure was standardized by using control tissue. JSB-1 mouse antibody (ab3366; AbCam, Cambridge, United Kingdom) was used for P-gp detection (1:50 dilution) and anti-MRP1 mouse antibody MRPm5 (ab24102; AbCam) was used for MRP1 detection (1:100 dilution).

The immunohistochemistry analysis was performed by an experienced (13 years) lung histopathologist (P.C.). The healthy lung tissue around the pathologic cancerous tissue or bullous lung in patients with spontaneous pneumothorax was clearly identified. The immunohistochemical expression of P-gp and MRP1 in the healthy bronchoalveolar epithelial tissue was graded semiquantitatively (27) by the histopathologist based on the strength of immunostaining and the number of cells staining for the protein per high-power field. The grading included four categories: negative = grade 0, weak = grade 1, moderate = grade 2, and strong = grade 3.

The immunohistochemistry analyses (P.C.) and the inhaled ^{99m}Tc -sestamibi clearance analyses were performed independently by H.K.M. and a nonauthor, blinded to each other's findings.

Statistical Analysis

Repeatability of the inhaled ^{99m}Tc -sestamibi clearance technique was assessed by using Bland-Altman analysis by testing agreement between the test and retest values. Mean inhaled

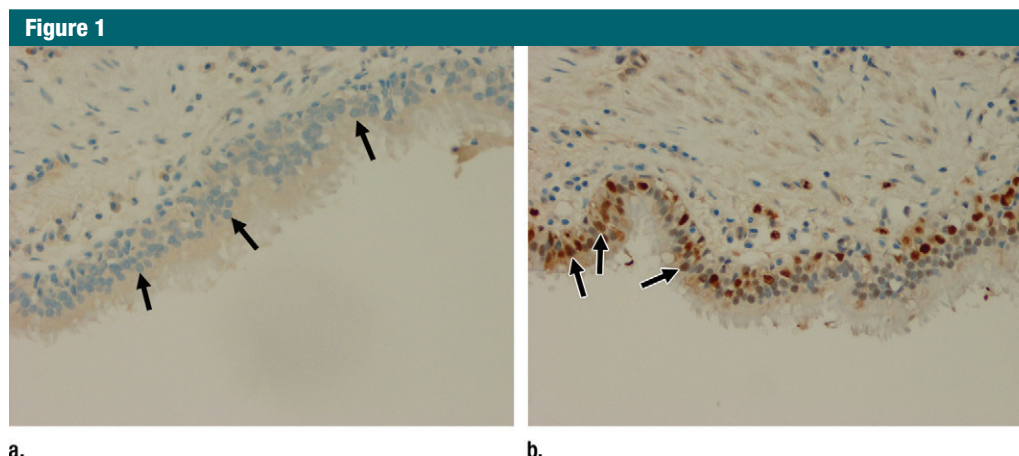


Figure 1: Photomicrographs (immunohistochemistry of P-gp and MRP1) of immunostaining of healthy bronchoalveolar epithelium in a patient demonstrate (a) negative P-gp staining with very little or no brown staining (arrows point to blue negative staining lining cells of the bronchoalveolar epithelium) and (b) intense brown staining for MRP1 expression (arrows point to brown positive staining lining cells of the bronchoalveolar epithelium).

^{99m}Tc -sestamibi clearance values and standard deviation were calculated. Parametric testing using Student *t* test and a nonparametric testing using Mann-Whitney test and a Spearman rank correlation test were applied to the data comparing the clearance half-time to the immunohistochemistry grading. Spearman ρ and concordance correlation coefficient test were used to test the correlation between the individual lung clearances and the mean clearance times assessed by the two reviewers.

Results

Tissue Immunohistochemistry

At immunohistochemical analysis of the lung tissue in 13 participants, the expression of P-gp within the normal bronchoalveolar epithelial tissue was negative (grade 0) (Fig 1a) in 11 patients and only weakly positive (grade 1) in two patients. MRP1 expression was demonstrated within the normal bronchoalveolar epithelium in 12 of 13 patients (Fig 1b) and was negative in only one patient. Five patients demonstrated grade 1 MRP1 expression, while six patients demonstrated grade 2 and one patient demonstrated grade 3 expression. The patients were divided into two groups:

those with low-grade expression, 0 and 1, and those with higher grade expression, 2 and 3, as in previous study (27).

Analysis of Inhaled ^{99m}Tc -Sestamibi Clearance

The clearance of inhaled ^{99m}Tc -sestamibi for individual lungs was obtained and compared. A Spearman ρ and concordance correlation coefficient test confirmed an excellent correlation between the right and left lung clearances in the first study and the repeat study ($P < .00$, $R = 0.9634$, and correlation coefficient = 0.9901).

Analysis of clearance of inhaled ^{99m}Tc -sestamibi from the lungs of the participants was significantly delayed in seven patients demonstrating upregulated MRP1 (grade 2–3, $T_{1/2} = 149 \text{ min} \pm 28$) compared with six patients expressing low MRP1 levels (grade 0–1, $T_{1/2} = 105 \text{ min} \pm 20$), Student *t* test $P = .008$ and Mann Whitney *U* test $P < .05$ (Fig 2a, 2b).

A further Spearman rank order test confirmed a significant correlation between the four different grades of immunohistochemical staining versus clearance of inhaled ^{99m}Tc -sestamibi from the lungs ($P = .0001$). The mean clearance of inhaled sestamibi from individual patients and the respective MRP1 expression grading are presented in the Table.

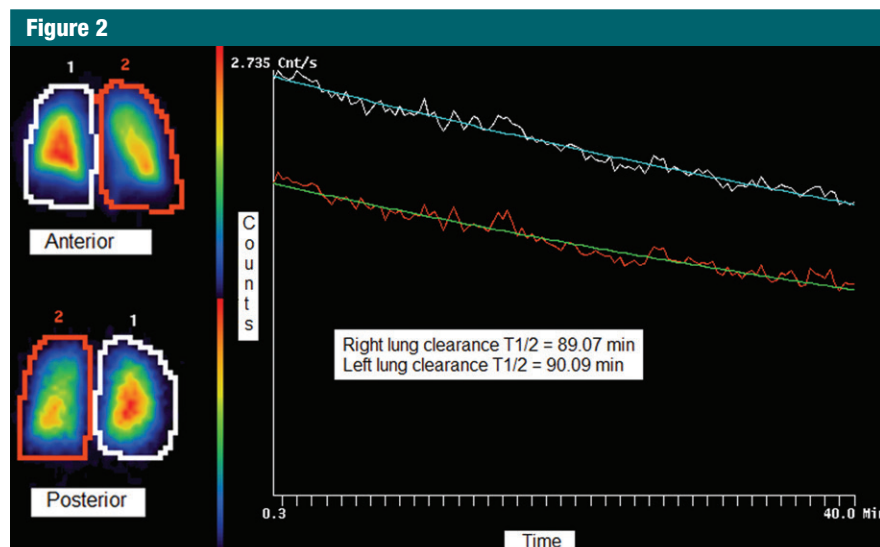
Repeatability of Inhaled ^{99m}Tc -Sestamibi Clearance Technique

Test-retest results in five participants who underwent repeat inhaled ^{99m}Tc -sestamibi clearance study demonstrated good agreement between the two measurements. Bland-Altman analysis revealed a percentage mean difference between test and retest clearance half-times (bias) of 3.23% and a percentage standard deviation of the difference (precision) of 3.9% (Fig 3).

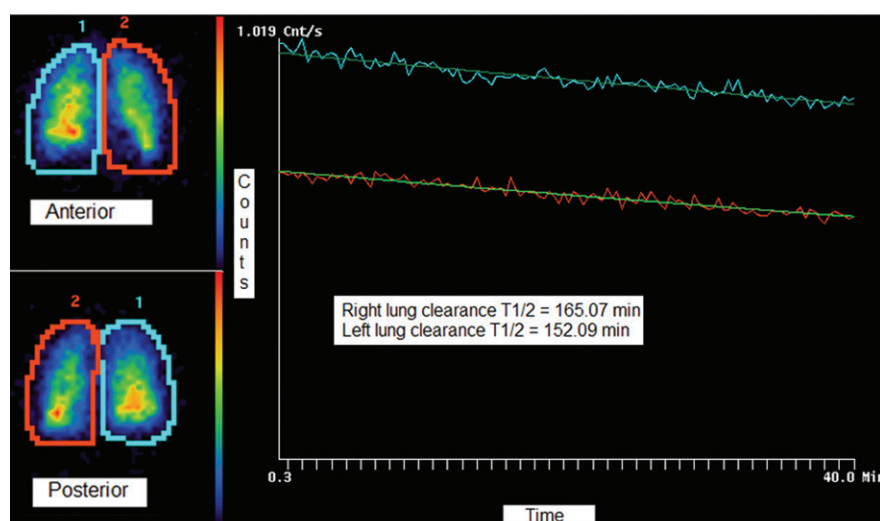
A second experienced nuclear medicine reviewer analyzed the individual right and left lung clearances in five patients independent of reviewer 1. Spearman ρ analysis of the individual lung clearances between the first and second reviewer revealed a statistically significant relation ($R = 0.97265$, two-tailed $P < .001$, and concordance correlation coefficient of 0.9865).

Discussion

The expression of P-gp and MRP1 in the human lung (28) suggests that these transporters may play an important role in the protection against endogenous or exogenous toxic compounds entering the lung. The delivery of inhaled drugs to reach their site of action may also be affected by the presence and activity of many cellular drug transporters (29). However, there



a.



b.

Figure 2: Inhaled ^{99m}Tc -sestamibi clearance in (a) a patient with low MRP1 expression and demonstrating a faster clearance time and in (b) a patient with high MRP1 expression and demonstrating prolonged clearance time.

remains a debate about the role of P-gp as a major drug transporter in human lungs. The current opinion is that P-gp is mainly expressed in the larger tracheobronchial airway epithelium, while there is no significant expression in bronchoalveolar epithelium (30).

Given the fact that MRP1 expression is higher in the lung compared with other solid organs, decreased or increased functional MRP1 expression may have a high impact on development and/or progression of lung diseases

and protection against air pollution and inhaled toxic substances, such as those present in cigarette smoke (31). Indeed, Van der deen et al observed lower MRP1 expression in patients with chronic airway disease and postulated that the lower expression of MRP1 or the down-regulation of MRP1 may result in an increased vulnerability to, and subsequent development of, chronic airway disease (31).

Imaging the kinetics of known drug transporter substrates across the

Individual Mean Inhaled Sestamibi Clearance Compared with the Histologic Grading

| Mean Clearance (min) | MRP1 Expression Grade |
|----------------------|-----------------------|
| 75.5 | 0 |
| 123 | 1 |
| 110.5 | 1 |
| 83.5 | 1 |
| 119 | 1 |
| 117.5 | 1 |
| 134.5 | 2 |
| 110 | 2 |
| 175.5 | 2 |
| 163 | 2 |
| 123 | 2 |
| 149.5 | 2 |
| 185.5 | 3 |

alveolocapillary membrane by using an inhaled clearance technique provides a model to directly interrogate the effects of drug transporters on a large variety of inhaled pulmonary therapeutic agents, such as corticosteroids and sympathomimetics that may be substrates for these transporters in the lung or modulate their activity. Functional inhalation studies would also provide an opportunity to demonstrate the effects of inhaled toxic substances, as postulated by van der Deen et al (31).

Inhaled ^{99m}Tc -sestamibi clearance is a functional imaging technique that was first described by Ruparel et al in 2008 (25). They studied the clearance of inhaled ^{99m}Tc -sestamibi in healthy human volunteers and patients with chronic obstructive pulmonary disease (COPD). They observed significant differences (> 20%) in inhaled ^{99m}Tc -sestamibi clearance rates between smokers and nonsmokers, while no significant difference was seen between healthy volunteers compared with COPD patients. They postulated that the delayed clearance of ^{99m}Tc -sestamibi across the alveolocapillary barrier was regulated by active binding and efflux of ^{99m}Tc -sestamibi by P-gp expressed in the lung tissue (26). They also postulated that delayed ^{99m}Tc -sestamibi clearance in smokers compared with nonsmokers

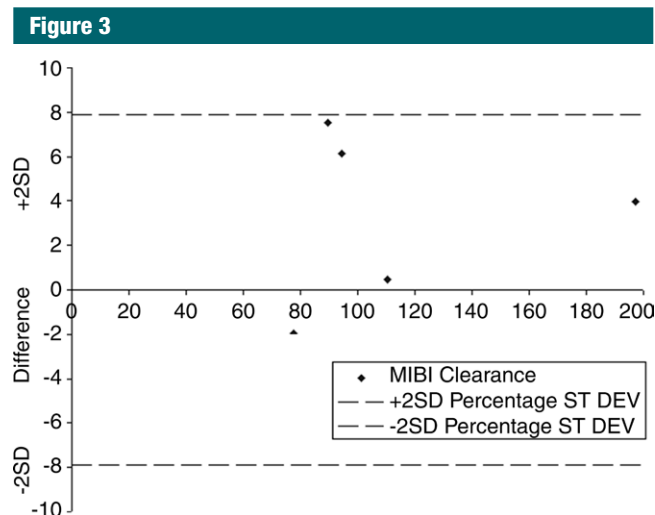


Figure 3: Bland-Altman analysis demonstrates excellent agreement between test and retest inhaled ^{99m}Tc -sestamibi (MIBI) clearance rates. $ST\ DEV$ = standard deviation.

was due to the active efflux of inhaled ^{99m}Tc -sestamibi mediated by increased P-gp expression in the alveolar epithelium, pumping it back into the air-spaces (25). Interestingly, this group showed no difference between smokers and nonsmokers with respect to the lung clearance rate of inhaled ^{99m}Tc -tetrofosmin (26). There was, however, no immunohistochemical correlation available to confirm the postulation.

In the present study, very little or no P-gp expression was identified, while in contrast, there was clear MRP1 expression noted at immunohistochemical analysis of these healthy lung tissue. Furthermore, we demonstrate a significant delay in the clearance of inhaled ^{99m}Tc -sestamibi, an established MRP1 substrate, from the lungs in patients with higher MRP1 expression (grade 2 or 3, $n = 7$) compared with those with low or no MRP1 expression (grade 0 or 1, $n = 6$).

Interestingly, we observed that all seven patients expressing higher MRP1 levels were also smokers and correspondingly demonstrated prolonged inhaled ^{99m}Tc -sestamibi clearance time. While the up-regulation of MRP1 in metaplastic lung epithelium following exposure to cigarette smoke has been observed by van der Deen et al (31), the current study results are similar to the observations of

Ruparelia et al (25), who also observed prolonged inhaled ^{99m}Tc -sestamibi clearance in smokers compared with nonsmokers, although they did not have immunohistochemical confirmation.

While immunohistochemistry is regarded as the reference standard for determination of MRP1 expression in lung epithelium, the technique is limited by heterogeneous expression of drug transporters in the lung tissue. The demonstration of expression is dependent on antibody selection and also remains tissue-dependent (13). Immunoreactivity is not specific, which could result in false-positive results. Other potential problems include sampling errors, tissue fixation effects (8), and failure of protein expression to translate to function. In contrast, functional imaging using radiolabeled drug transporter substrates represents an attractive alternative for the in vivo measurement of functional expression and moreover is independent of histologic sampling errors.

However, on review of the literature, functional imaging studies demonstrating the role of MRP1 in lungs remains very limited. Apart from a single animal study by Okamura et al (32), who demonstrated MRP1 as the prime drug transporter across the lung epithelium with P-gp playing no significant role, there is a lack of functional

imaging studies demonstrating the role of MRP1 in lungs.

This study has limitations. The number of patients was small, which makes interpretation of the results, although significant, at best a relation rather than causal effect. We also did not look at possible regional variation of the clearance values and did not analyze the clearance specifically related to tumor tissue compared with the normal lung tissue. However, given the size of the pathologic finding, which was 1–4 cm maximum in all cases, it was considered too small to attempt to correlate spatially with regions of interest on the dynamic images, which are subject to patient motion and respiratory motion, which would affect the spatial correlation. In addition, due to the influence of noise in the dynamic images, the use of small regions of interest would also lead to errors related to this analysis.

Nonetheless, this study is the first in vivo study, to our knowledge, that demonstrates a correlation between lung MRP1 expression and inhaled ^{99m}Tc -sestamibi clearance, which is a safe (radiation exposure < 0.5 mSv) and a repeatable technique. The results of this study do raise the need for a further prospective study with larger numbers to assess if these results could be replicated. Further studies should also look at the effect of specific MRP1 blockers to address the causal relation and assess the effect of other covariables, including smoking status, effect of age and sex on the clearance of inhaled ^{99m}Tc -sestamibi.

In conclusion, inhaled ^{99m}Tc -sestamibi clearance presents a safe and repeatable technique demonstrating a correlation between bronchoalveolar expression of MRP1 and clearance of inhaled ^{99m}Tc -sestamibi. There was no substantial P-gp expression identified in the normal lung tissue. Further research is needed to assess the factors influencing the clearance of inhaled ^{99m}Tc -sestamibi and determine the potential applicability of these findings in clinical practice.

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no relevant relationships. **L.L.** disclosed no relevant relationships. **J.R.B.** disclosed no relevant relationships. **A.M.P.** disclosed no relevant relationships.

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